Purification of bacterially-expressed chick NSD3-SET that is active in an *in vitro* methyltransferase assay

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Daniel Alan Kretzschmar

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Adviser: Laura S. Gammill, Ph.D.

December, 2014

Acknowledgements

First, I would especially like to thank my advisor, Dr. Laura Gammill, for her mentorship and guidance throughout my time in the lab. I truly appreciate your tireless patience, clear insight into both scientific and personal areas of life, and steadfast willingness to help a 'non-traditional' student find his path in whatever way you can.

Many thanks to the other members of the Gammill Lab that I have had the pleasure of working with: Dr. Julaine Roffers-Agarwal, Dr. Bridget Jacques-Fricke, Dr. Katie Vermilion, Dr. Corinne Fairchild, Kevin Lidberg, and Joe Conway, for all your friendly advice and technical support, including offering helpful comments on a lot of nightmarish practice talks.

I would also like to thank all the past and present members of my committee; Dr. Vivian Bardwell, Dr. Jeff Simon, Dr. Lorene Lanier, Dr. Daniel Voytas, and Dr. Paul Letourneau, for all their scientific input.

Finally, thanks to all the fellow Developmental Biology Center members which have supplied technical advice, reagents, and/or a bench corner for me to work on, especially: Dr. Aswathy Rai, Dr. Marcus Vargas, Ellen Miller, and Dr. Micah Gearhart.

Abstract

The lysine methyltransferase NSD3, which belongs to a family of histone H3 lysine 36 methyltransferases that are conserved among vertebrates and overexpressed in many cancers, is independently required for both neural crest cell specification and migration in the developing chick embryo. Since cytoplasmic protein lysine methylation is also required for neural crest cell migration in chick, one possibility is that NSD3 methylates non-histone substrates. To test this hypothesis, it is necessary to purify NSD3 protein that is active in a methyltransferase assay, in order to evaluate candidate substrates individually and on protein arrays. In this thesis, I describe a detailed method for high-yield purification of a chick NSD3 catalytic fragment, followed by a chick NSD3 methyltransferase assay with which to test for activity on polynucleosomes and/or candidate non-histone substrates.

Table of Contents

Acknowledgements i
Abstract ii
List of Tables iv
List of Figures v
List of Abbreviations vi

Chapter 1: Introduction 1
1.1 The *in vitro* methyltransferase assay is an indispensable test of KMT function and/or specificity 2
1.2 The NSD family of lysine methyltransferases 4
1.3 Chick NSD3 is an attractive candidate for non-histone target discovery 6

Chapter 2: An optimized procedure for high-yield purification of catalytically active chick NSD3-SET 8
2.1 Procedure notes & overview 9
2.1.1 Notes about the procedure 9
2.1.2 Overview of the procedure 12
2.2 Materials 14
2.2.1 Reagents 14
2.2.2 Equipment 16
2.3 Procedure: NSD3-SET purification 17
2.3.1 Bacterial culture and NSD3-SET expression 17
2.3.2 Crude analysis of NSD3-SET expression 19
2.3.3 Cell lysis, Ni-NTA column purification and CFD buffer exchange 21
2.4 Procedure: NSD3-SET *in vitro* methyltransferase assay 30
2.5 Anticipated results 33

References 39

List of Tables

Table 1. Volumes of protease inhibitors to add, given frequently used buffer volumes **20**

List of Figures

Figure 1. Lysis of bacterial cultures with a French press **23**

Figure 2. Ni-NTA Superflow column purification **28**

Figure 3. Purification of chick NSD3-SET has been optimized to >90% purity. **35**

Figure 4. Purified chick NSD3-SET is active in a methyltransferase assay. **37**

List of Abbreviations

CFD: centrifugal filter device

E1, E2: first and second eluate

H3K36: histone H3, lysine 36

IPTG: isopropyl-β-D-thiogalactopyranoside

KMT: lysine methyltransferase

MTase: methyltransferase

NCC: neural crest cell

Ni-NTA: nickel-nitrilotriacetic acid

NPI: sodium phosphate imidazole

NSD: nuclear receptor-binding, SET domain-containing protein (1, 2, 3)

NSD3-SET: NSD3 catalytic fragment, amino acids 1021-1320

PI: protease inhibitor

PMSF: phenylmethanesulfonyl fluoride

PRC: Polycomb repressive complex

^{3}H-SAM: S-adenosyl-L- [methyl-^{3}H] methionine

SET: Su(var)3-9, Enhancer-of-zeste and Trithorax

TCEP: Tris-(2-Carboxyethyl)phosphine

TLCK: Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride

Chapter 1

Introduction

1.1 The *in vitro* methyltransferase assay is an indispensable test of KMT function and/or substrate specificity

Ever since the discovery of the evolutionarily conserved protein lysine methyltransferase Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain in *Drosophila* (Tschiersch et al., 1994), and the realization that histone lysine methylation profoundly influences the regulation of eukaryotic gene expression (Lachner and Jenuwein, 2002), proteins containing a SET domain have been almost axiomatically referred to as 'histone methyltransferases' (Rea et al., 2000; Kondo, 2014). However, since many SET domain-containing lysine methyltransferases such as NSD1, G9a, EZH2, SET7/9 and many others have also been shown to methylate non-histone proteins (Huang and Berger, 2008; Lu et al., 2010), the term is something of a misnomer that presupposes a singular biological role for SET enzymes. As evidence builds to indicate that non-histone protein methylation affects target subcellular localization, stability, and interactions with other proteins or nucleic acids (Lee and Stallcup, 2009), the more appropriately generalized terms 'protein lysine methyltransferase' or simply 'lysine methyltransferase' (KMT), have come into more common usage (Clark, 2013; Lanouette, 2014). Given this emerging awareness, the development of experimental tools to characterize the substrate specificity and full functionality of each KMT is becoming increasingly paramount.

A fundamental means to test putative substrates of any SET domain-containing KMT is an *in vitro* methyltransferase (MTase) assay. Typically, this

consists of incubating a KMT with the radiolabeled methyl donor S-adenosyl-L-[methyl-^{3}H] methionine (^{3}H-SAM) and substrate(s), then performing SDS-PAGE, Western blot, and autoradiography. Most frequently, MTase assays are used to evaluate individual candidate substrates, identify target lysines through mutational analysis, and/or define MTase activity for SET domain-containing KMTs, from fruit fly E(Z) to human NSD1 (Müller et al., 2002; Kudithipudi et al., 2014). These studies can eventually yield not only an in-depth understanding of a KMT and its preferred substrates in isolation, but also variation in activity (and by extension, biological function) based on association with other endogenous factors, which are often necessary for maximal MTase activity *in vitro* (Ketel et al., 2005).

When a MTase assay is adapted for a broader scale using arrays, many proteins can be simultaneously evaluated as potential substrates. For instance, the human KMT G9a and ^{3}H-SAM were applied to a peptide array based on single variants of histone H3 N-terminal sequence, yielding 2 strongly methylated candidate non-histone targets (Rathert et al., 2008). More recently, an improved substrate array consisting of over 9,000 human proteins expressed in insect cells and printed in duplicate (ProtoArray®), was used to find over 100 novel non-histone targets of SETD6 (Levy et al., 2011). Certainly, whether candidate substrates are arrayed for an unbiased assessment of KMT specificity, or tested in isolation for detailed biochemical analyses, a methyltransferase assay is an essential tool to investigate KMT non-histone protein targets.

1.2 The NSD family of lysine methyltransferases

A group of closely-related KMTs of particular relevance to this thesis are the nuclear receptor-binding, SET domain-containing (NSD) family of enzymes, which are conserved throughout the vertebrate lineage, and are oncogenic when overexpressed in humans (Lucio-Eterovic and Carpenter, 2011; Morishita and di Luccio, 2011;). Actually, NSD1 is the only one of these known to bind to nuclear receptors (Huang et al., 1998), but the official names of the others are cumbersome (WHSC1, WHSC1L1), so I will henceforth refer to them as NSD2 and NSD3.

All three of these proteins are highly similar within a 700 amino acid region that includes the core SET domain and the associated pre-SET and post-SET domains (Angrand 2001), and share several protein-protein and/or protein-DNA interaction domains. Despite an initial controversy over the histone lysine residue specificity of the human NSDs (Kim et al., 2006), it was thoroughly investigated using bacterially-expressed 300 amino acid catalytic NSD fragments and different types of histone substrates, revealing that human NSDs are histone H3 lysine 36 (H3K36) dimethylases on nucleosomes (Li et al., 2009). In that study, the Reinberg group also showed full-length NSD2 had the exact same H3K36 specificity on nucleosomes, even when purified in complex with endogenous factors from HT1080 human fibrosarcoma cells.

Recently, human NSD family enzyme kinetics were characterized in detail, and it was found that a shared basic region within the post-SET domain is largely

responsible for the different specificities on various histone substrates (Allali-Hassani et al., 2014). H3K36 specificity on nucleosomes was confirmed using nearly identical catalytic fragments as before, while a set of truncations were found to reduce or abolish activity. Thus, at least for the human NSD family, it appears substrate specificity is retained regardless of whether full-length enzyme or the catalytic fragment described in (Li et al., 2009) is used in a MTase assay.

In addition to all three human NSDs, homologs found in higher eukaryotes share this H3K36 dimethylation specificity on chromatin. In *Drosophila*, H3K36 dimethylation by the KMT dMes-4 is thought to play a role in nucleosome positioning and RNA splicing (Bell et al., 2007; Lhoumaud et al., 2014). The *C. elegans* NSD homolog MES-4 is also required for H3K36 dimethylation, and its histone H3 specificity is retained in an *in vitro* MTase assay using bacterially-expressed enzyme (Bender et al., 2006).

Strikingly, the NSD family is also highly conserved throughout the vertebrate lineage, and especially mammals and birds (NCBI HomoloGene). Thus, it is imperative that the complete *in vivo* function of these KMTs is investigated using robust experimental tools, such as are available in mice and chick.

1.3 Chick NSD3 is an attractive candidate for non-histone target discovery

Interestingly, we found two chick KMTs in a screen for factors involved in the vertebrate-specific process of neural crest development: NSD3 and SETD2 (Gammill and Bronner-Fraser, 2002; Adams et al., 2008). Additionally, we also found other components of the SAM-dependent *in vivo* methylation cycle, including DNMT3B, KDM5A and SAHH. Given these results, our lab has been working to understand the full impact of methylation on neural crest development. This is especially crucial because prenatal supplementation with the methylation-associated compound folic acid is widely used to prevent neural tube and neural crest-related birth defects, yet the mechanistic basis for this effect remains unknown (Wehby and Murray, 2011). Moreover, efforts to understand methylation are urgently needed because both broad-spectrum and selective methylation inhibitors are used as chemotherapy agents (though none specifically inhibit the NSDs; Kaniskan et al., 2014).

Indeed, since NCCs are a migratory, multipotent and highly invasive mesenchymal cell type, similar in both gene expression and behavioral phenotype to metastatic cancers (Gupta et al., 2005; Thiery et al., 2009), elucidating NSD3's vertebrate-specific functional role in migratory cells could have far-reaching significance. Regarding neural crest-derived metastatic melanoma in particular, there is increasing evidence to support the model that oncogenesis involves reactivation of the NCC embryonic transcriptional program (Bailey et al., 2012).

While NSD3's role as a transcription-coupled chromatin modifier is beginning to emerge (Fang et al., 2010), multiple lines of evidence suggest NSD3-mediated non-histone protein methylation could regulate NCC migration in a novel manner, independent of its role as a H3K36 dimethylase. First, taking note of the comparatively weak H3K36 activity of both NSD1 and NSD3 *in vitro* (Li et al., 2009), and that NSD1 is known to methylate the non-histone p65 subunit of NF-κB (Lu et al., 2010), it is reasonable to think NSD3 also methylates non-histone substrates. Furthermore, not only have we found that NSD3 is required for both NCC specification and migration, and that these roles are distinct in developmental time (Jacques-Fricke and Gammill, 2014), we find NSD3 localized in the cytoplasm via immunostaining in both chick NCCs and human C8161 melanoma cells (data not shown). Even more strikingly, we find that methylation of 6 lysine residues of EF1α1, an abundant cytoplasmic translational elongation factor found at the leading edge of motile cells (Condeelis and Singer, 2005), is required for NCC migration in chick (Vermillion et al., 2014).

Clearly, establishing a chick NSD3 methyltransferase assay to assess candidate non-histone substrates, whether singly in a tube or affixed to a ProtoArray®, is a critical step toward investigating the role of NSD3 methylation in both neural crest development and related cancers.

Chapter 2

An optimized procedure for high-yield purification of catalytically active chick NSD3-SET

2.1 Procedure notes & overview

2.1.1 Notes about the procedure

The steps contained in this procedure have been adapted and assembled from a variety of sources, including the published study revealing human NSD family H3K36 specificity on DNA-wrapped nucleosomes (Li et al., 2009), Ni-NTA Superflow column product literature (Qiagen), and the technical expertise of fellow University of Minnesota Developmental Biology Center lab members (see **Acknowledgements**) and Life Technologies Technical Staff.

I originally purified 3xFLAG-tagged full-length chick NSD3 from a baculoviral insect cell system, but found early preparations to be inactive. Rather than troubleshoot that system, and/or express full-length NSD3 in human cells and attempt to co-purify endogenous complexes necessary for robust activity (as was performed for full-length human NSD2; Li et al., 2009), I opted for the relatively expedient method of obtaining catalytically active chick NSD3 for use in a MTase assay; that is, a bacterially-expressed 300 amino acid NSD3 catalytic fragment as per (Li et al., 2009). This fragment corresponds with 95% identity[1] to amino acids 1021-1320 of human NSD3, and although it includes several domains in addition to the core SET domain, I will henceforth refer to it as NSD3-SET.

A key difference from the Reinberg lab NSD3-SET purification method was that we chose to affix an N-terminal 6xHis-tag to NSD3-SET and perform Ni-NTA column purification, rather than a GST-tag with glutathione columns. This decision was mainly due to the very small size of the 6xHis-tag, which we assumed to be less likely to cause undesirable effects on NSD3-SET purification and/or MTase activity, versus the 26 kDa GST-tag.

Imidazole competes with the binding of the 6xHis tag to the Ni-NTA column, and is therefore used at lower concentrations to reduce the binding of

---

[1] (NCBI BLAST alignment: *G. gallus* XP_001232891.1 residues 1021-1320 vs. *H. sapiens* NP_075447.1)

[2] Alternatively, storage of NSD3-SET purified preparations in liquid N_2 may extend the

histidine-rich contaminating proteins, and for elution at 250 mM. However, 250 mM imidazole is inhibitory to KMT activity (Life Technologies Technical Staff, personal communication), and therefore buffer exchange must be performed prior to using the purified NSD3-SET in a methyltransferase (MTase) assay. Typically, an overnight dialysis at 4°C is performed, then the eluate is concentrated at room temperature using 'spin columns' or centrifugal filter devices (CFDs). However, since both dialysis membranes and CFDs have a molecular weight cutoff rating, I tried to shorten the overall time for the procedure by omitting the overnight dialysis, in favor of simply performing the buffer exchange within the CFD itself. This is accomplished by adding fresh imidazole-free buffer to the concentrated sample, spinning again, and repeating this for several rounds. Surprisingly, though this method slightly increases room-temperature sample handling time within the CFD, it did not result in any noticeable adverse effects. In fact, opting for MicroSep™ CFD-mediated buffer exchange improved sample purity while maintaining NSD3-SET activity in the downstream MTase assay **(Fig. 3, Fig. 4)**.

A key concern with native, non-denaturing protein purification is maintaining solubility. Often a bacterially-expressed protein that is normally soluble can become entrapped in inclusion bodies, and precipitate along with cellular debris when clearing the lysate via centrifugation. I determined that reducing the culture temperature to 17°C immediately before IPTG induction (as in Li et al., 2009) maintained NSD3-SET solubility, likely due to slowing the rate of protein expression and preventing its sequestration within inclusion bodies.

Later in the procedural workflow, while performing CFD-mediated buffer exchange, another issue with NSD3-SET solubility arose. I resolved this by removing glycerol from the elution buffer, which was also advantageous for other reasons. First, glycerol is inhibitory to MTase activity at assay concentrations above ~15% (Aswathy Rai, personal communication), and so a limited amount can be added to a MTase assay. When NSD3-SET is prepared without glycerol,

a slightly less concentrated sample can be tested, and/or an increased amount of enzyme can be added to the assay. Second, because Life Technologies screens ProtoArrays® using a Tris-based reaction buffer without glycerol, glycerol-free purification conforms to their standards.

2.1.2 Overview of the procedure

I. A pET24b+ expression plasmid containing N-terminally 6xHis-tagged chick NSD3-SET is freshly transformed into BL21 (DE3) *E. Coli* cells.

II. Starter cultures are grown from single colonies at 37°C, and expanded to 1.5 L total volume. Near the end of log-phase growth, the temperature is reduced to 17°C, and NSD3-SET is expressed under IPTG induction for 18 hours.

III. Cells are harvested by centrifugation, and pellets are stored at -80°C for a minimum of 1 hour.

- **PAUSE POINT:** Pellets may be stored at -80° C for 4 days, or possibly longer, but catalytic activity may suffer.

IV. Robust expression of NSD3-SET is confirmed via SDS-PAGE & Coomassie staining, comparing uninduced vs. induced crude cell lysates.

V. The NSD3-SET expression cultures are resuspended in low-imidazole buffer (NPI-10) at 4°C and lysed with a pre-chilled French press.

VI. The cell lysate is ‘cleared’ via centrifugation at 4°C, and further cleared with 5 µm syringe filters, removing particulates which may clog the Ni-NTA column.

VII. Still at 4°C, NSD3-SET contained in the cell lysate is bound to an equilibrated Ni-NTA Superflow column, washed with buffers of increasing stringency (NPI-20 and NPI-80) and eluted with 250 mM imidazole.

VIII. The samples are brought to room temperature and buffer exchange is performed within CFDs, using several rounds of centrifugation and addition of fresh imidazole-free buffer, and a final centrifugation to concentrate the sample.

IX. Aliquots of the purified, concentrated NSD3-SET preparation are snap-frozen in liquid N_2 and stored[2] at -80°C, save ~5 µl kept on ice for quality control analysis.

- **PAUSE POINT:** Purified KMT aliquots may be stored long-term at -80°C; however, it was determined that NSD3-SET activity rapidly declines within 2-4 weeks. Thus, quality control testing and downstream experiments (i.e., ProtoArray®) should be performed within one week or less, if possible.

X. The concentration of the NSD3-SET preparation is determined with the Qubit Protein Assay.[3]

XI. The purity of the NSD3-SET preparation is assessed with SDS-PAGE, followed by Coomassie staining.

XII. NSD3-SET catalytic activity is tested with the MTase assay using polynucleosome control substrate. NSD3-SET methylation of any purified candidate substrates in-hand, or complex mixtures of chick proteins (i.e., tissue/cell lysates) may be tested simultaneously also.

---

[2] Alternatively, storage of NSD3-SET purified preparations in liquid N_2 may extend the half-life of its MTase activity, though this remains untested.

[3] Though a Bradford protein assay may be used, the Qubit® Protein Assay requires as little as 1 µl of purified sample to reliably determine concentration.

2.2 Materials

2.2.1 Reagents

- Chick NSD3-SET catalytic fragment cDNA, cloned with an N-terminal 6xHis tag into pET-24b+ *E. Coli* expression vector, mini-prepped and sequence-verified.
- Kanamycin 1000X stock solution, 37.5 mg/mL
- Super LB: 2X LB mix + 2.0 g glucose per liter
- Sodium phosphate imidazole (NPI) buffers:
  - NPI-10: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole
  - NPI-20: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole
  - NPI-80: 50 mM NaH_2PO_4, 300 mM NaCl, 80 mM imidazole
  - NPI-250: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole
- Imidazole-free KMT storage buffer: 50 mM Tris pH 9.0, 5 mM $MgCl_2$, 1 mM DTT (Life Technologies recommended)
- Protease inhibitor stock solutions
  - 10^4 base PIs (10,000x base protease inhibitors): In 1 mL DMSO, add 10 mg leupeptin (Sigma L2023-10MG), 10 mg chymostatin (Sigma C7268-5MG, Qty. 2), 10 mg pepstatin A (Sigma P4265-5MG, Qty. 2) and 5 mg antipain (Sigma A6191-5MG). Aliquot 100 µl per tube and store at -20°C.
  - PMSF 100 mM (phenylmethanesulfonyl fluoride, Sigma P7626-250MG[4]): Dissolve 0.1742 g in 10 mL isopropanol. Aliquot 1 mL per tube and store at -20°C.
  - DTT 1 M (dithiothreitol, Sigma D9779[5]): Dissolve 1.5425 g in 10 mL 0.01 M sodium acetate, pH 5.2. Filter sterilize, aliquot 1 mL per tube and store at -20°C.

[4] Gammill Lab location: room temperature dessicator.
[5] Gammill Lab location: dessicator in deli 4°C.

- TLCK 50 mg/mL (Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride, Sigma A1153-10MG): Add 2 mL HCl 1 mM to 100 mg bottle, aliquot 1 mL per tube and store at -20°C.
- Benzamidine 0.1 M (Sigma 434760-5G): Dissolve in sterile H_2O, aliquot 1 mL per tube and store at -20°C.
- Aprotinin 10 mg/mL (Sigma A1153-10MG): Add 1 mL sterile H_2O to bottle, aliquot 100 µl per tube and **store at 4°C**.

- GelCode™ Blue Safe Coomassie protein stain (ThermoFisher 24594)
- Qubit® Protein Assay Kit (Life Technologies Q3321)
- 2X MTase assay buffer (Simon Lab):

  24 mM HEPES pH 7.9
  0.48 mM EDTA
  24% glycerol
  8 mM DTT
  5 mM $MgCl_2$
  60 mM KCl

- Histone H3-specific positive control KMT, e.g., *Drosophila* PRC (Simon Lab)
- Amido Black Staining Solution 2X (Sigma A8181-1EA)
- EN^{3}HANCE™ autoradiography spray (Perkin Elmer 6NE970C)
- Immobilon™-P transfer membrane (Sigma P2938-1ROL)
- Polynucleosomes (1 mg/mL) purified from HeLa cells (Simon Lab; Biovest International, Inc.)
- Towbin transfer buffer with SDS (25 mM Tris, 192 mM glycine, 10% methanol, and 0.01% SDS)

2.2.2 Equipment

- Incubated/refrigerated orbital shaker[6] (Forma™ 4580) or equivalent
- Spectrophotometer, for taking OD_{600} readings
- Incubated centrifuge[7] (Beckman J2-MC) or equivalent
- FRENCH® pressure cell press[8] (Thermo Electron FA-078) or equivalent
- Acrodisc® 32 mm Syringe Filter with 5 µm Supor® Membrane (Pall Corp. 4650)
- PureLink™ Nucleic Acid Purification Rack (Life Technologies K2100-13) or equivalent
- Ni-NTA Superflow Column (Qiagen 30622). Store upright at 4°C.
- Pipet-Aid® serological pipette controller (USA Scientific 4440-1000) or equivalent
- MicroSep™ Advance Centrifugal Device, 30 kDa molecular weight cutoff (Pall Corp. MCP03046)[9]. Store at RT.
- Qubit® 2.0 Flourometer (Life Technologies Q32866)
- Tritium-labeled S-adenosyl-L-[methyl-^{3}H] methionine
- Scintillation fluid and scintillation vials
- Lift-Away decontaminant (RPI International 114002)

[6] Large shaker in Moos 5-140, behind door near Kawakami Lab cryostat
[7] Centrifuge in Bardwell Lab Moos 5-145
[8] Lange Lab French press components are stored at 4°C in MCB 7-159
[9] Amicon® Ultra-4 30 kDa MW cutoff (Millipore UFC803008) was compared in parallel and found to be inferior to the MicroSep™ CFD.

2.3 Procedure: NSD3-SET purification

2.3.1 Bacterial culture and NSD3-SET expression

- ***CRITICAL:*** *Start each purification from a freshly-transformed culture, as the expression plasmid w/insert is unstable in BL21 cells.*

1. Transform expression plasmid into BL21 (DE3) *E. Coli* competent cells.
    i. Thaw competent BL21 cells on ice, up to 30 minutes.
    ii. Add 25 µl cells to 1 µl plasmid (assuming ~200 ng/µl DNA concentration) and gently pipette up and down 2-3X to mix.
    iii. Incubate 30 minutes on ice.
    iv. Heat shock at 42°C for 1 minute, then incubate on ice for 2 minutes.
    v. Add 475 µl SOC at RT.
    vi. Recover 1-2 hours at 37°C w/shaking ~250 rpm.
    vii. Streak out 50 µl of each transformation on an LB-kan plate using the micropipette tip or a sterile toothpick, and incubate O/N at 37°C.

- ***CRITICAL:*** *BL21 cells are high-efficiency; spreading the full reaction will result in colony overgrowth.*

2. Inoculate several pre-warmed 3 mL LB-kan starter cultures by picking single colonies. Incubate at 37°C w/shaking ~250 rpm for 4 hours.
3. While the starter cultures are incubating, pre-warm the large incubated/refrigerated orbital shaker to 37°C. Adjust the setting such that the thermometer inside actually reads 37°C (i.e., incubator setting of ~35.9°C).
4. Prepare two bottles of Super LB (750 mL each) by autoclaving, and add 750 µl kanamycin (final concentration 37.5 µg/mL). Save 1 mL as a spectrophotometer 'blank' for OD_{600} readings.
5. Pour 750 mL Super LB-kan into two clean, autoclaved 2 L Erlenmeyer flasks, and place the culture flasks in the incubated/refrigerated shaker.

Allow to equilibrate to 37°C. *(Hint: tin foil suffices as a vented culture flask cap.)*

6. Inoculate each flask containing 750 mL Super LB-kan with one 3 mL starter culture each. Incubate at 37°C w/shaking ~250 rpm for 4 hours.
7. Begin checking OD_{600} every 30-60 minutes. Once OD_{600} reaches 2.2-2.8, reduce the incubated/refrigerated shaker temperature setting to approximately 15.9°C, such that the thermometer inside the shaker stably reads 17°C.
   - ***CRITICAL:*** *Ensure that the temperature inside the culture chamber is stable at 17°C before induction of NSD3-SET expression.*
8. Withdraw 0.5-1.0 mL of each culture for a non-induced expression control (see **2.3.2**). Spin these samples down, remove the media, and save the pellets at -20°C.
9. Induce protein expression by adding 375 µl 1M IPTG to each culture flask (final concentration 0.5 mM). Incubate 18 hours at 17°C w/shaking ~250 rpm.
10. Just before harvesting cultures, withdraw 0.5-1.0 mL for an induced expression control (see **2.3.2**). Spin these samples down, remove the media, and save the pellets at -20°C.
11. Harvest cultures by centrifugation at 4°C to pellet the bacterial cells. Spin several rounds in two 250 mL plastic bottles with a JA-17 rotor @ 7,000 rpm, pouring off the media after each spin. Freeze cell pellets at -80°C for a minimum of 1 hour.
    - **PAUSE POINT**: Cell pellets containing the expressed His-NSD3-SET can be stored at -80°C for 4 days, or possibly longer, but catalytic activity may suffer.

2.3.2 Crude analysis of NSD3-SET expression

12. Thaw cell pellets (from **Step 8** and **Step 10**) and resuspend in 75 µl PBS.
13. Add 25 µl 4X SDS-PAGE sample buffer w/BME and vortex 30 seconds.
14. Spin 10 minutes at 12,000 X g.
15. Boil samples 5-10 minutes in heat block.
16. Load 16 µl of each sample in a 4-12% polyacrylamide gel, and electrophorese at 80V until past the 4% stacking gel, then ~1 hour at 100V.
17. Remove the gel and wash it in nanopure water, 3 x 5 minutes.
18. Stain the gel for 1-2 hours with shaking in GelCode Blue Safe Coomassie stain.
19. Destain gel in nanopure water for 2-3 hours to O/N. Folding up a Kimwipe and putting it along the inner side of the container helps speed up destaining.

final X	5 mL	10 mL	15 mL	20 mL	25 mL	50 mL	stock X
	For full PI cocktail to given volume, add:						
2X base PIs	1 µl	2 µl	3 µl	4 µl	5 µl	10 µl	10^4 base PIs
2X aprotinin	5 µl	10 µl	15 µl	20 µl	25 µl	50 µl	10 mg/mL aprotinin
1 mM benzamidine	5 µl	10 µl	15 µl	20 µl	25 µl	50 µl	1 M benzamidine
50 µg/mL TLCK	5 µl	10 µl	15 µl	20 µl	25 µl	50 µl	50 mg/mL TLCK
1 mM DTT[10]	5 µl	10 µl	15 µl	20 µl	25 µl	50 µl	1 M DTT
0.4 mM PMSF	20 µl	40 µl	60 µl	80 µl	100 µl	200 µl	100 mM PMSF

Table 1. Volumes of protease inhibitors to add, given frequently used buffer volumes

[10] The DTT concentration is reduced from 2 mM to 1 mM, which differs from the Simon Lab PI cocktail, originally to comply with Ni-NTA Superflow column buffer recommendations (Ni-NTA Superflow BioRobot Handbook, Qiagen 09/2002). Later, it was discovered that the maximum recommended DTT concentration is actually 10 mM (Important Note for Ni-NTA users, Qiagen 05/2008). Regardless, 1 mM DTT was used in the PI cocktails for the catalytically active 12-27-13 preparation **(Fig. 3, FIg. 4)**, so that is what is given here.

2.3.3 Cell lysis, Ni-NTA column purification and CFD buffer exchange

20. Thaw cell pellets for 15 minutes at RT, then place on ice. Add 40 mL ice-cold resuspension buffer with protease inhibitors (NPI-10+PIs, see **Table 1**) to each 250 mL bottle, and resuspend by vortexing and/or pipetting up and down. Incubate on ice 30 minutes before lysing cells.
21. Transfer each cell suspension to a pre-chilled 50 mL conical tube. Lyse the cells using a French press **(Fig. 1)**, maintaining ~900-1000 psi, **(Fig. 1G)** and running each sample through the press 3x at a dropwise rate **(Fig. 1F)**. Collect the lysate with a clean pre-chilled 50 mL tube each time. Detailed steps are as follows:
    i. Inspect the components, especially the O-rings on the cap and piston **(Fig. 1A-iv, -v)** and the plastic flow valve seat bead **(Fig. 1B)**. Replace if noticeably damaged.
    ii. Check the press itself. If it's not lowered all the way, turn the hydraulic pump on, select DOWN **(Fig 1G-i, -ii)**, wait until it's lowered, then turn the pump off (it's best not to let the pump run when it's not in motion). Open the safety bar if it's in the way.
    iii. Screw the outlet nozzle into the cylinder and finger-tighten. Also screw in the flow valve and gently ensure that it is closed, but do not overtighten, as that will deform the plastic bead **(Fig. 1B)**.
    iv. Insert the piston into the cylinder, and place the assembly into the inverted filling stand. Either prop the piston or hold it to prevent it from falling out, if necessary **(Fig. 1C)**.
    v. Pour ~40 mL cell suspension into the cylinder and put the cap on the cylinder. Note that this will create internal pressure and push the piston out slightly (with the valve closed, as it should be) or eject cell suspension from the outlet (with the valve open).

vi. Holding the capped end of the cylinder with one hand and the piston with the other, invert the assembly and place it into the press. Close the safety bar and tighten at least one of the set screws **(Fig. 1D-ii)**.

vii. If necessary, turn the piston handle such that it won't contact the safety set screw when the press is fully compressed **(Fig. 1E)**. Gently ensure the flow valve is closed, but don't overtighten.

viii. Begin raising the press ram by turning on the pump and selecting MED, then immediately check the pressure reading **(Fig. 1G-i, -ii, -iii)**. If it is extremely higher or lower than ~450, adjust the knob **(Fig. 1G-iv)**. Very soon, the top of the press will contact the piston and begin to apply pressure.

ix. Prepare to collect the lysate by holding the clean, pre-chilled 50 mL tube as shown **(Fig. 1F)**.

x. Open the flow valve slowly and begin collecting lysate at a dropwise rate. Check the gauge pressure again, and adjust it with the knob again, if necessary.

xi. Maintain dropwise collection. The valve position needed for this will vary during operation. Be prepared for a sputter of debris-ridden lysate at the end of the press cycle.

xii. Repeat 2x for each ~40 mL cell suspension (3 press cycles total), collecting in a clean, pre-chilled 50 mL tube each time.

- ***CRITICAL:*** *Keep lysates on ice as much as possible. Also, chill the cylinder assembly on ice for ~10-15 minutes before lysing the next ~40 mL cell suspension.*

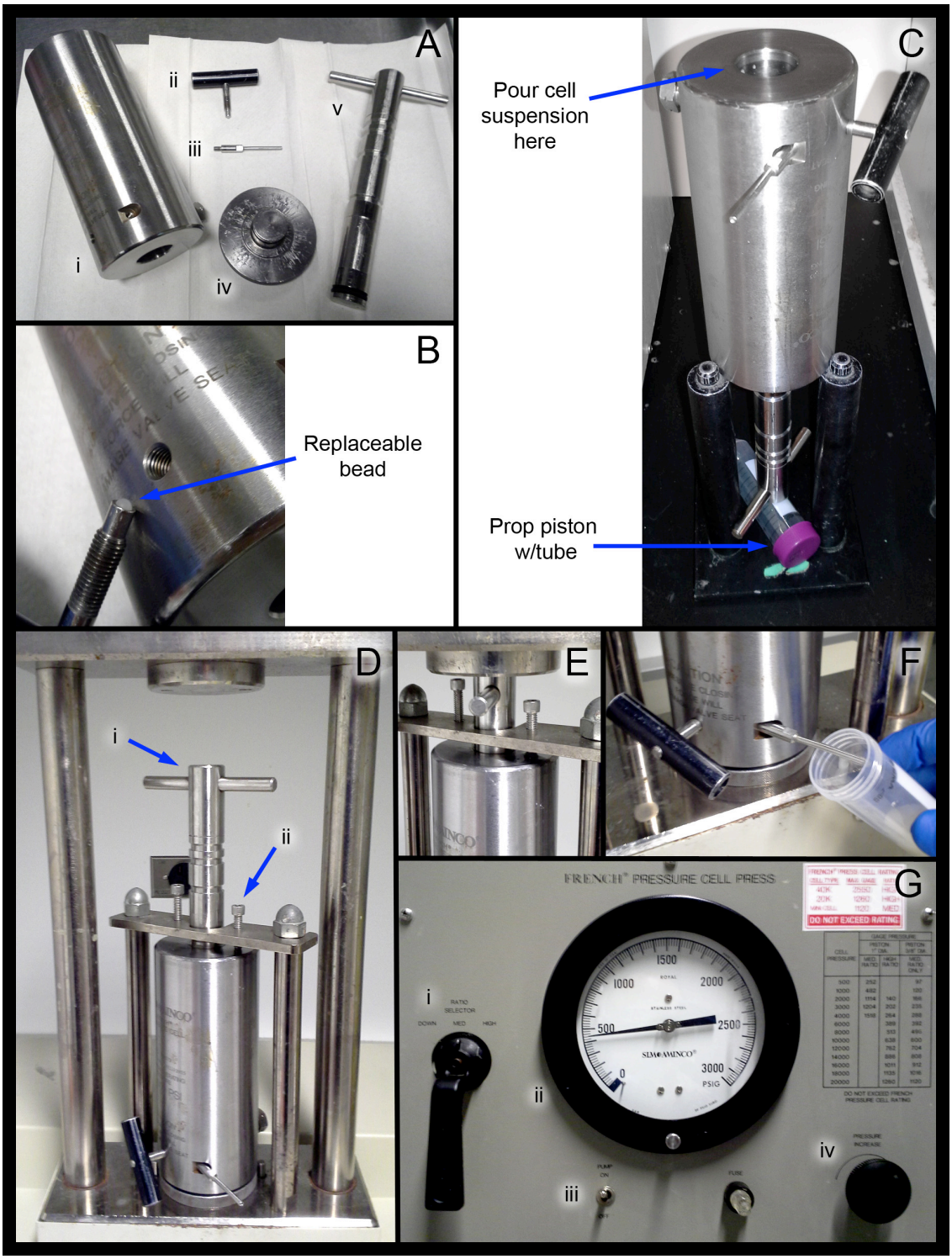
A
ii
v
iii
i
iv
B
Replaceable bead
C
Pour cell suspension here
Prop piston w/tube
D
i
ii
E
F
G
FRENCH® PRESSURE CELL PRESS
DO NOT EXCEED RATING
i
ii
iii
iv

Figure 1. Lysis of bacterial cultures with a French press

A) French press components requiring assembly: i) cylinder; ii) flow valve; iii) outlet nozzle; iv) cylinder cap; v) piston. **B)** Plastic valve seat bead must be replaced if deformed, or the valve will not fully close. **C)** Press components semi-assembled on the inverted stand, ready to add cell suspension. The piston must be propped or manually held to prevent it from falling out when the cap is put on. **D)** Assembled press. (i) Turn the piston handle 90° from position shown, and (ii) tighten at least one of the safety set screws. **E)** Piston at lower limit of travel; note the handle orientation prevents contact with the safety screw. **F)** During press operation, adjust valve to keep a dropwise flow rate. **G)** Press control panel: i) Press operating lever. The “DOWN” position lowers the hydraulic ram, while “MED” compresses the piston. Do not use the “HIGH” setting. ii) Gauge pressure reading for maintaining ~900-1000 psi cell pressure[11]. iii) Hydraulic pump on/off switch. iv) Pressure adjustment knob.

[11] The chart on the control panel indicates a gauge pressure of 482 equals 1000 psi cell pressure, using the 1” diameter piston and selecting the MED ratio.

22. Clear the cell lysate by centrifugation at 5,000 x g at 4°C for 1-2 hours[12]. This pellets the majority of cellular debris, and the insoluble protein fraction.
23. When centrifuging is complete, remove any floating cellular debris from the tubes of cell lysate with a P1000 micropipette.
24. Further clear the cell lysates by filtering with 5 µm syringe filters. Several syringe filters may be required per 50 mL lysate, as they clog up with debris. Save the insoluble pellet at -80°C, as it may be needed for troubleshooting.
    - ***CRITICAL:*** *Depress the syringe slowly to minimize foaming, as this can denature proteins.*
25. Set up the Ni-NTA Superflow column and purification rack in a 4°C room.
    - ***CRITICAL:*** *Always use a new column for each purification batch. In my hands, column reuse had undesirable downstream effects on purity.*
26. Check that the bluish Ni-NTA resin is settled properly in the Superflow column **(Fig. 2A-ii)**. If it was not properly stored upright, bluish resin will be visible in the column filling chamber **(Fig. 2A-i)**. Let the column stand vertically in the rack until the resin is settled, if necessary.
27. Cut the tip of the column to open the outlet **(Fig. 2A-iii)**.
28. Remove the white cap, and allow the storage buffer to drain by gravity flow **(Fig. 2B)** or apply mild vacuum pressure **(Fig. 2C)**.
    - ***CRITICAL:*** *Never let the column run dry while applying vacuum pressure. Instead, remove the vacuum tubing and allow the column to finish draining by gravity flow. The column will not run dry by gravity flow.*
29. Equilibrate the Ni-NTA column with 10 mL pre-chilled resuspension buffer (NPI-10+PIs), and allow the column to drain by gravity flow.
30. Transfer ~10 mL cleared lysate to the Ni-NTA column loading reservoir. Allow to drain by gravity flow **(Fig. 2D)**. Continue to add the rest of the

[12] The Bardwell Lab JA-17 rotor with 'partially supported' 50 mL conical tubes limits this spin to 5,000 x g, or 4,000 rpm. Otherwise, higher speeds could be used.

cleared lysate[13]. Collect several drops of column outflow at least once during this step (and steps **31-32**) for later analysis. Sampling of additional time points may help with any troubleshooting that may be necessary.

31. Wash with 2 x 10 mL buffer NPI-20+PIs. Apply mild vacuum pressure and/or allow the column to drain completely by gravity flow.
32. Perform an additional stringent wash by adding 5 mL buffer NPI-80+PIs. Apply mild vacuum pressure and/or allow the column to drain completely by gravity flow.
33. Elute the bound NSD3-SET by adding 3 mL buffer NPI-250+PIs, collecting the flow-through in a clean 15 mL conical tube. Allow the column to drain completely by gravity flow.
34. Perform a second elution by adding another 3 mL buffer NPI-250+PIs, and collecting in a new tube. Allow to drain completely by gravity flow.
35. Allow the eluates to reach RT by incubating ~10 min on the bench. Transfer the first and second eluate (E1, E2) each to a 30 kDa MW cutoff MicroSep™ centrifugal filter device (CFD).
36. Centrifuge E1 and E2 at 2,000 rpm for 15-30 minutes. The sample volumes should be reduced to 0.5-1 mL, with E2 the lower of the two, due to a lower NSD3-SET concentration. Empty the lower collection tubes of the CFDs. Add imidazole-free buffer (50 mM Tris pH 9.0, 5 mM $MgCl_2$, +PIs) to fill the upper part of the CFD (to 5 mL).
37. Repeat the previous step approximately three times. Track the volume of the concentrated eluates after each spin and the volume of fresh buffer added. Be careful not to over-concentrate the first elution, which contains the majority of the purified NSD3-SET.

---

[13] The 1.5 mL of Ni-NTA resin in the column has a binding capacity of 75 mg, which is more than enough to accommodate the expressed protein from 1.5 L total culture volume. Thus, ~80 mL of cleared lysate (total) is allowed to flow through a single column.

- ***CRITICAL:*** *Inspect the concentrated eluates carefully (especially E1) after each spin for signs of precipitation. If cloudiness is present near the bottom of the filter cartridge, and buffer exchange is incomplete, add 1-2 mL fresh buffer and pipet mix before centrifuging again.*

38. Buffer exchange is complete when the two 3 mL eluates have been diluted 1:50 or better. (An imidazole concentration of 5 mM is acceptable, as the preparation will be diluted in a MTase assay.)
39. Centrifuge one final time to concentrate the sample, if necessary, and remove the sample with a syringe.
    - ***CRITICAL:*** *Minimize foaming when withdrawing the sample with a syringe, as this can denature proteins.*
40. Aliquot as desired, snap-freeze in liquid N_2, and store at -80°C[14]. A small aliquot (~5 µl) may be kept on ice for immediate testing with the MTase assay, and/or other quality control testing (see below).
    - **PAUSE POINT**: It was determined that NSD3-SET MTase activity rapidly declines within 2-4 weeks when purified under these conditions. Thus, quality control testing and downstream experiments (i.e., ProtoArray®) should be performed as soon as possible.
41. Determine the concentration of the NSD3-SET purified preparation with the Qubit Protein Assay, according to the manufacturer's instructions.
42. Perform SDS-PAGE followed by Coomassie staining to assess final NSD3-SET sample purity, and the efficiency of the column purification, if desired.

[14] Storage of NSD3-SET in liquid N_2 may increase the shelf life of its MTase activity, though this possibility remains untested.

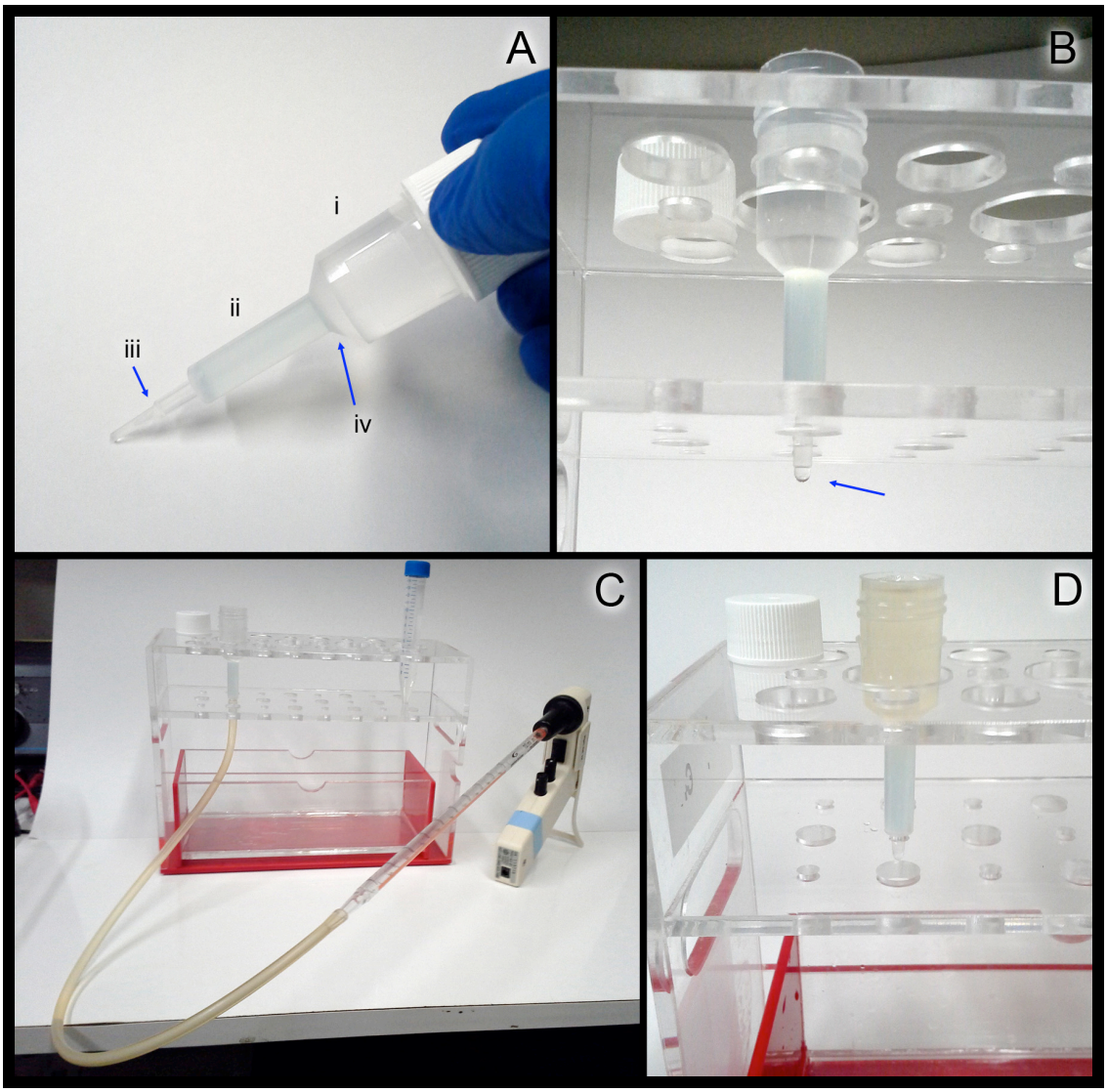
A
i
ii
iii
iv
B
C
D

Figure 2. Ni-NTA Superflow column purification

A) The Ni-NTA Superflow column: i) 10 mL column loading reservoir; ii) column packed with 1.5 mL Ni-NTA resin; iii) column outlet (cut at arrow); iv) column filling chamber neck, note the bluish Ni-NTA resin has already migrated from the column after being held at an angle for ~30 seconds. **B)** View from below the column while in the purification rack, with equilibration buffer (NPI-10+PIs) flowing out dropwise by gravity flow. **C)** Assembled apparatus for applying mild vacuum pressure[15] to the column to increase flow rate: latex tubing, a 10 mL serological pipette and a Pipet-Aid®. Apply pressure with the Pipet-Aid® such that the column loading reservoir is near-empty in approximately 4 minutes (or longer). **DO NOT** let the column run dry under vacuum pressure. **D)** Column loaded with cleared lysate.

[15] Ni-NTA Superflow column recommended vacuum pressure is -10 mbar, while the recommended flow rate is 1-3 mL/minute (QIAexpressionist, Qiagen 06/2003).

2.4 Procedure: *in vitro* methyltransferase assay with purified NSD3-SET

43. Set up each 20 µl MTase assay reaction as given below. Typically, use 2 µl (20 nM final concentration) for the histone H3 positive control enzyme complex PRC (Simon Lab). For initial testing of purified NSD3-SET, test three enzyme concentrations (i.e., 100 nM, 200 nM, 400 nM). If testing substrates other than polynucleosomes, start with 500 ng substrate per reaction. First, assemble all reaction components, except the tritiated SAM.

    10 µl 2X MTase buffer
    2 µl polynucleosomes (1 mg/mL)
    X µl KMT (NSD3-SET, or positive control PRC)
    X µl sterile distilled H_2O (to 20 µl)
    1 µl ^{3}H-SAM (0.001 mCi)

44. Move behind the plastic shield in the radioactivity area. Add the 1 µl tritiated SAM to each reaction.

45. Incubate the reactions for 1-1.5 hours in a 30°C heat block. Meanwhile, pour a 4-15% SDS-PAGE gel.

46. Stop the MTase reactions by adding 5 µl 4x SDS sample buffer to each.

47. Load each full reaction in the gel (Step **45**), and electrophorese for 1 hour at 30 mA.

48. Remove gel from the running apparatus and place it in Towbin transfer buffer. Incubate at RT for at least 15 minutes. Meanwhile, dispose of all radioactive waste properly, and prepare the Immobilon-P membrane for transfer.

49. Cut the Immobilon-P membrane to size. Place it methanol for 15 seconds. Transfer into distilled H_2O for 2 minutes (do not submerge). Place membrane in Towbin buffer for at least 5 minutes.

50. Prepare the transfer stack. Soak filter paper in Towbin buffer. Build the stack from the bottom up: filter paper, Immobilon-P membrane, gel, and filter paper. Roll out any air bubbles.
51. Transfer at 20V for 20-30 minutes. When done with the transfer, place all components of the stack (except the membrane) in the tritium solid waste.
52. Allow the membrane to dry for 1-5 minutes. Place it into Amido Black Stain, which allows visualization of all transferred proteins. Incubate at RT for 5 minutes.
53. Transfer membrane to 100% methanol. Wash quickly 3x to remove the excess Amido Black stain.
54. Tape the membrane down to a large piece of filter paper.
55. In the fume hood, spray the membrane with EN^{3}HANCE spray. Cover completely, but try not to saturate. Allow the membrane to dry, which takes 10-15 minutes.
56. Rotate the filter paper with attached membrane 90°, and spray again. Repeat two more times, such that the membrane has been sprayed in all four orientations.
57. Place the membrane in a cassette (preferably the same one which is always used for MTase assays—as the EN^{3}HANCE spray has an unpleasant odor). Tape it down, along with a piece of filter paper with luminescent writing on it, which serves as a fiducial marker.
58. In the darkroom, place a single-sided film emulsion side-down on the membrane. Do not use intensifying screens. Expose the film overnight at -80°C, or for up to a week.
59. Develop film while it is still frozen; do not let it thaw. A new film may be exposed for a week or longer, if a shorter exposure has already been completed.

60. For final clean-up, take Q-tip smears of pipettors, the bench in various places, the shaker, and anything possibly contaminated with radioactivity. Place the smears in 3 mL scintillation fluid in a scintillation vial, and count against a blank on an open window in the scintillation counter. Decontaminate with Lift-Away as necessary.

61. Log the volume of ^{3}H-SAM used and from which aliquot. Also, log the amount of radioactive waste as follows: 5% solid waste (0.00005 mCi); 95% liquid waste (0.00095 mCi).

2.5 Anticipated results

This optimized procedure yields ~900 µg of >90% pure N-terminally 6xHis-tagged NSD3-SET **(Fig. 3)**, which methylates histone H3 of polynucleosome substrate in an *in vitro* methyltransferase assay **(Fig. 4)**. While early permutations of the procedure also yielded catalytically active NSD3-SET **(Fig. 4, purified 3-27-13)**, the protocol detailed here achieves NSD3-SET purity and yield that is suitable for non-histone target discovery via the ProtoArray® (Life Technologies Technical Staff, personal communication). However, the weak activity of NSD3-SET (as compared to the robust H3 methylation catalyzed by *Drosophila* PRC), and the very short period in which NSD3-SET retains even this low level of MTase activity (1-2 weeks, data not shown) raises several concerns.

First, a key consideration is whether a large fraction of bacterially-expressed NSD3-SET consists of misfolded proteins or aggregates, leading to its observed lackluster MTase activity. Native PAGE and/or high-resolution size exclusion chromatography, such as with a Superdex 200 column, could rule this out and ensure purified NSD3-SET homogeneity (GE Healthcare Life Sciences). However, it is important to note that bacterially-expressed human NSD3-SET also showed weak histone H3 methylating activity, despite an extra cleanup or 'polishing' step with a Superdex 200 column and pooling of the resulting samples (Li et al., 2009). Indeed, this is in keeping with the mounting evidence for NSD3 as a KMT with additional, non-histone substrates *in vivo* **(1.3)**.

A second significant drawback to NSD3-SET purified in this manner is its loss of catalytic activity within a few weeks, thus rendering the successive testing of individual candidate substrates (such as chick EF1α1) somewhat impractical. Although this short KMT shelf life also complicates the logistics of candidate target discovery with ProtoArray® methyltransferase substrate profiling, it does not negate such an array-based experiment's potential as a critical step toward revealing novel, non-histone substrates of NSD3, if any.

Importantly, a thorough kinetic characterization of human NSD catalytic fragment activity *in vitro* was recently published, which revealed that the use of TCEP instead of DTT as the disulfide reducing agent resulted in a six-fold increase in NSD3-SET activity on polynucleosomes (Allali-Hassani et al., 2014). However, a different MTase assay was used than the method described here, and TCEP was also the reducing agent in buffers throughout their purification procedure. Therefore, it is unclear whether simply using TCEP instead of DTT in the reaction buffer will achieve the same dramatic improvement in chick NSD3-SET methylation of polynucleosomes. Furthermore, TCEP is not particularly stable in sodium phosphate buffers (Thermo Fisher Scientific), so making full use of this insight may require re-optimization of the procedure.

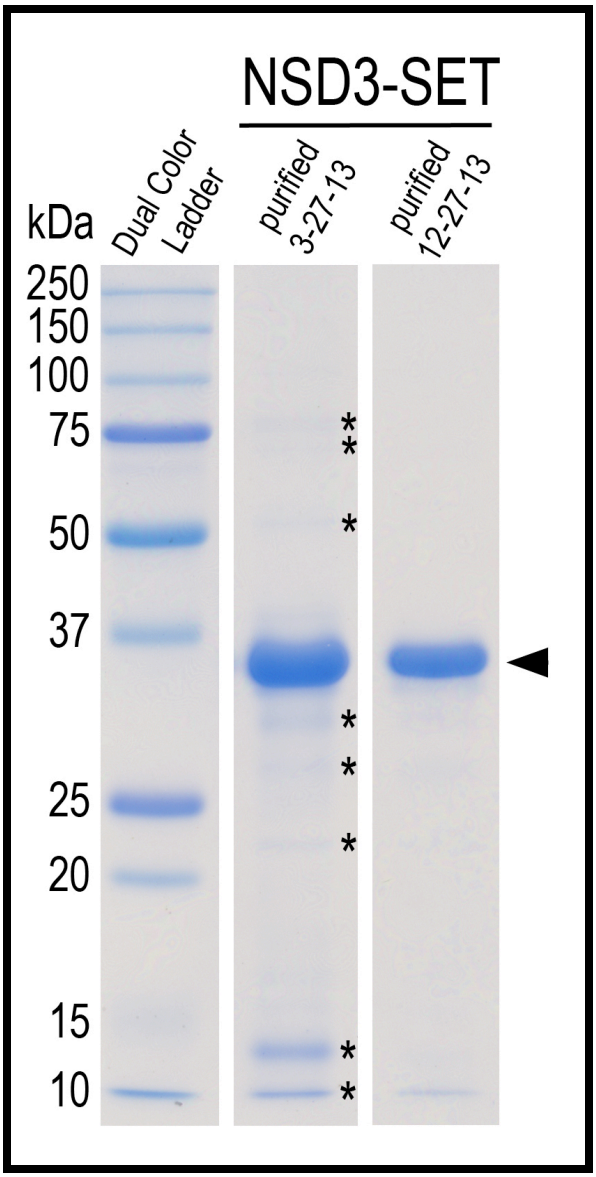
NSD3-SET
kDa
Dual Color Ladder
purified 3-27-13
purified 12-27-13
250
150
100
75
50
37
25
20
15
10

Figure 3. Purification of chick NSD3-SET has been optimized to >90% purity. Peptide contaminants (asterisks) were found in the low-yield NSD3-SET preparation purified 3-27-13 (previously active in a MTase assay), and were even more prominent in later scaled-up preparations. The purification procedure was then optimized for high yield (>900 µg) at greater than 90% purity (purified 12-27-13).

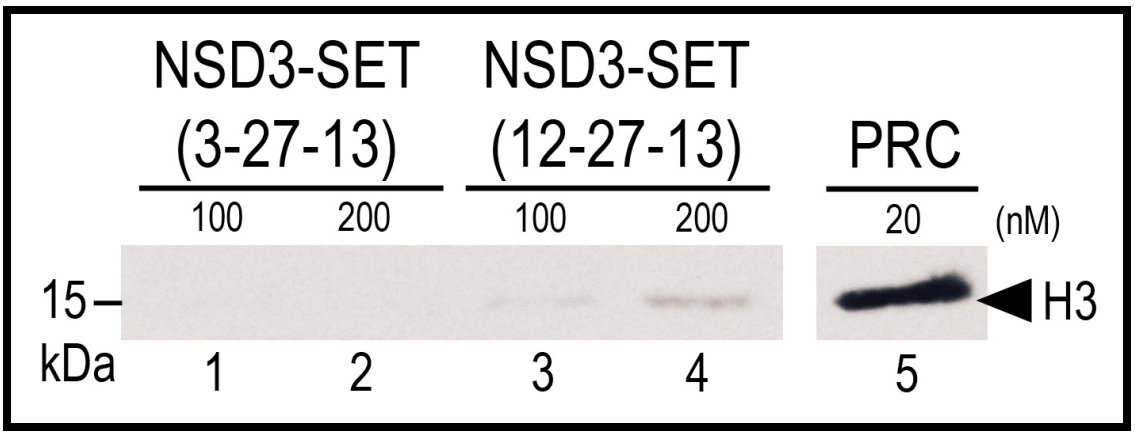
NSD3-SET
(3-27-13)
NSD3-SET
(12-27-13)
PRC
100
200
100
200
20
(nM)
15–
kDa
H3
1
2
3
4
5

Figure 4. Purified chick NSD3-SET is active in a methyltransferase assay. After optimizing recombinant chick NSD3-SET purification for large-scale culture in bacteria, methyltransferase activity on histone H3 (arrowhead; all lanes) was restored (purified 12-27-13; lanes 3 & 4), although it is very weak compared to Drosophila PRC complex (lane 5) and rapidly declines. Previously-active NSD3-SET purified 3-27-13, no longer shows detectable activity (lanes 1 & 2).

References

Adams, M.S., Gammill, L.S., and Bronner-Fraser, M. (2008). Discovery of transcription factors and other candidate regulators of neural crest development. Dev. Dyn. *237*, 1021–1033.

Allali-Hassani, A., Kuznetsova, E., Hajian, T., Wu, H., Dombrovski, L., Li, Y., Gräslund, S., Arrowsmith, C.H., Schapira, M., and Vedadi, M. (2014). A Basic Post-SET Extension of NSDs Is Essential for Nucleosome Binding In Vitro. J. Biomol. Screen., 1-8.

Angrand, P.O., Apiou, F., Stewart, A.F., Dutrillaux, B., Losson, R., and Chambon, P. (2001). NSD3, a new SET domain-containing gene, maps to 8p12 and is amplified in human breast cancer cell lines. Genomics *74*, 79–88.

Bailey, C.M., Morrison, J.A., and Kulesa, P.M. (2012). Melanoma revives an embryonic migration program to promote plasticity and invasion. Pigment Cell Melanoma Res. *25*, 573–583.

Bell, O., Wirbelauer, C., Hild, M., Scharf, A.N.D., Schwaiger, M., MacAlpine, D.M., Zilbermann, F., van Leeuwen, F., Bell, S.P., Imhof, A., et al. (2007). Localized H3K36 methylation states define histone H4K16 acetylation during transcriptional elongation in Drosophila. EMBO J. *26*, 4974–4984.

Bender, L.B., Suh, J., Carroll, C.R., Fong, Y., Fingerman, I.M., Briggs, S.D., Cao, R., Zhang, Y., Reinke, V., and Strome, S. (2006). MES-4: an autosome-associated histone methyltransferase that participates in silencing the X chromosomes in the C. elegans germ line. Development *133*, 3907–3917.

Clarke, S.G. (2013). Protein methylation at the surface and buried deep: thinking outside the histone box. Trends Biochem. Sci. *8*, 147-149.

Condeelis, J., and Singer, R.H. (2005). How and why does beta-actin mRNA target? Biol. Cell Auspices Eur. Cell Biol. Organ. *97*, 97–110.

Fang, R., Barbera, A.J., Xu, Y., Rutenberg, M., Leonor, T., Bi, Q., Lan, F., Mei, P., Yuan, G.-C., Lian, C., et al. (2010). Human LSD2/KDM1b/AOF1 regulates gene transcription by modulating intragenic H3K4me2 methylation. Mol. Cell *39*, 222–233.

Gammill, L.S., and Bronner-Fraser, M. (2002). Genomic analysis of neural crest induction. Development *129*, 5731-5741.

Gupta, P.B., Kuperwasser, C., Brunet, J.-P., Ramaswamy, S., Kuo, W.-L., Gray, J.W., Naber, S.P., and Weinberg, R.A. (2005). The melanocyte differentiation program predisposes to metastasis following neoplastic transformation. Nat. Genet. *37*, 1047–1054.

Huang, J., and Berger, S.L. (2008). The emerging field of dynamic lysine methylation of non-histone proteins. Curr. Opin. Genet. Dev. *18*, 152–158.

Huang, N., vom Baur, E., Garnier, J.M., Lerouge, T., Vonesch, J.L., Lutz, Y., Chambon, P., and Losson, R. (1998). Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. EMBO J. *17*, 3398–3412.

Jacques-Fricke, B.T., and Gammill, L.S. (2014). Neural crest specification and migration independently require NSD3-related lysine methyltransferase activity. Mol. Biol. Cell. E13-12-0744 [Epub ahead of print].

Kaniskan, H.U., Konze, K.D., and Jin, J. (2014). Selective Inhibitors of Protein Methyltransferases. J. Med. Chem. [Epub ahead of print].

Ketel, C.S., Andersen, E.F., Vargas, M.L., Suh, J., Strome, S., and Simon, J.A. (2005). Subunit Contributions to Histone Methyltransferase Activities of Fly and Worm Polycomb Group Complexes. Mol. Cell. Biol. *25*, 6857–6868.

Kim, S.M., Kee, H.J., Eom, G.H., Choe, N.W., Kim, J.Y., Kim, Y.S., Kim, S.K., Kook, H., Kook, H., and Seo, S.B. (2006). Characterization of a novel WHSC1-associated SET domain protein with H3K4 and H3K27 methyltransferase activity. Biochem. Biophys. Res. Commun. *345*, 318–323.

Kondo, Y. (2014). Targeting histone methyltransferase EZH2 as cancer treatment. J. Biochem. (Tokyo) *156*, 249–257.

Kudithipudi, S., Lungu, C., Rathert, P., Happel, N., and Jeltsch, A. (2014). Substrate specificity analysis and novel substrates of the protein lysine methyltransferase NSD1. Chem. Biol. *21*, 226–237.

Lachner, M., and Jenuwein, T. (2002). The many faces of histone lysine methylation. Curr. Opin. Cell Biol. *14*, 286–298.

Lanouette, S., Mongeon, V., Figeys, D., and Couture, J.-F. (2014). The functional diversity of protein lysine methylation. Mol. Syst. Biol. *10*.

Lee, Y.-H., and Stallcup, M.R. (2009). Minireview: Protein Arginine Methylation of Nonhistone Proteins in Transcriptional Regulation. Mol. Endocrinol. *23*, 425–433.

Levy, D., Liu, C.L., Yang, Z., Newman, A.M., Alizadeh, A.A., Utz, P.J., and Gozani, O. (2011). A proteomic approach for the identification of novel lysine methyltransferase substrates. Epigenetics Chromatin *4*, 19.

Lhoumaud, P., Hennion, M., Gamot, A., Cuddapah, S., Queille, S., Liang, J., Micas, G., Morillon, P., Urbach, S., Bouchez, O., et al. (2014). Insulators recruit histone methyltransferase dMes4 to regulate chromatin of flanking genes. EMBO J. *33*, 1599–1613.

Li, Y., Trojer, P., Xu, C.F., Cheung, P., Kuo, A., Drury, W.J., Qiao, Q., Neubert, T.A., Xu, R.M., Gozani, O., et al. (2009). The target of the NSD family of histone lysine methyltransferases depends on the nature of the substrate. J. Biol. Chem. *284*, 34283–34295.

Lu, T., Jackson, M.W., Wang, B., Yang, M., Chance, M.R., Miyagi, M., Gudkov, A.V., and Stark, G.R. (2010). Regulation of NF-κB by NSD1/FBXL11-dependent reversible lysine methylation of p65. Proc. Natl. Acad. Sci. *107*, 46–51.

Lucio-Eterovic, A.K., and Carpenter, P.B. (2011). An open and shut case for the role of NSD proteins as oncogenes. Transcription *2*, 158–161.

Morishita, M., and di Luccio, E. (2011). Cancers and the NSD family of histone lysine methyltransferases. Biochim. Biophys. Acta BBA - Rev. Cancer *1816*, 158–163.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197–208.

Rathert, P., Dhayalan, A., Murakami, M., Zhang, X., Tamas, R., Jurkowska, R., Komatsu, Y., Shinkai, Y., Cheng, X., and Jeltsch, A. (2008). Protein lysine methyltransferase G9a acts on non-histone targets. Nat. Chem. Biol. *4*, 344–346.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., et al. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593–599.

Thiery, J.P. (2009). Metastasis: Alone or Together? Curr. Biol. *19*, R1121–R1123.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G., and Reuter, G. (1994). The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. EMBO J. *13*, 3822–3831.

Vermillion, K.L., Lidberg, K.A., and Gammill, L.S. (2014). Cytoplasmic protein methylation is essential for neural crest migration. J. Cell Biol. *204*, 95–109.

Wehby, G.L., and Murray, J.C. (2010). Folic acid and orofacial clefts: a review of the evidence. Oral Dis. *16*, 11–19.